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2018-10

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Rosa , E , Woestmann , L , Biere , A & Saastamoinen , M 2018 , ' A plant pathogen modulates the effects of secondary metabolites on the performance and immune function of an insect herbivore ' , Oikos , vol. 127 , no. 10 , pp. 1539-1549 . <https://doi.org/10.1111/oik.05437>

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<http://hdl.handle.net/10138/251510>

<https://doi.org/10.1111/oik.05437>

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## Research

### A plant pathogen modulates the effects of secondary metabolites on the performance and immune function of an insect herbivore

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#### Oikos

127: 1539–1549, 2018

doi: 10.1111/oik.05437

Subject Editor: Sara Magalhães

Editor-in-Chief: Dries Bonte

Accepted 7 May 2018



Host plant chemical composition critically shapes the performance of insect herbivores feeding on them. Some insects have become specialized on plant secondary metabolites, and even use them to their own advantage such as defense against predators. However, infection by plant pathogens can seriously alter the interaction between herbivores and their host plants. We tested whether the effects of the plant secondary metabolites, iridoid glycosides (IGs), on the performance and immune response of an insect herbivore are modulated by a plant pathogen. We used the IG-specialized Glanville fritillary butterfly *Melitaea cinxia*, its host plant *Plantago lanceolata*, and the naturally occurring plant pathogen, powdery mildew *Podosphaera plantaginis*, as model system. Pre-diapause larvae were fed on *P. lanceolata* host plants selected to contain either high or low IGs, in the presence or absence of powdery mildew. Larval performance was measured by growth rate, survival until diapause, and by investment in immunity. We assessed immunity after a bacterial challenge in terms of phenoloxidase (PO) activity and the expression of seven pre-selected insect immune genes (qPCR). We found that the beneficial effects of constitutive leaf IGs, that improved larval growth, were significantly reduced by mildew infection. Moreover, mildew presence downregulated one component of larval immune response (PO activity), suggesting a physiological cost of investment in immunity under suboptimal conditions. Yet, feeding on mildew-infected leaves caused an upregulation of two immune genes, lysozyme and prophenoloxidase. Our findings indicate that a plant pathogen can significantly modulate the effects of secondary metabolites on the growth of an insect herbivore. Furthermore, we show that a plant pathogen can induce contrasting effects on insect immune function. We suspect that the activation of the immune system toward a plant pathogen infection may be maladaptive, but the actual infectivity on the larvae should be tested.

Keywords: insect immunity, iridoid glycosides, Lepidoptera



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## Introduction

Plants harbor a diverse community of microbes and arthropod herbivores interacting with each other directly, or through the phenotypic changes they induce in the shared host (Hatcher 1995, Karban and Kuc 1999). Tripartite interactions critically shape the dynamics of plant- and insect-associated communities (Biere and Tack 2013, Tack and Dicke 2013). For example, insect herbivores can facilitate the spread of plant symbionts or pathogens by creating entry points into plant tissues through mechanical feeding damage (Biere and Bennett 2013) or by acting as vectors of microbial plant diseases (Kluth et al. 2002). Furthermore, insects can facilitate or hamper the performance of beneficial microbes and plant pathogens on their shared host by altering plant nutritional quality or by activating specific defense pathways (Lazebnik et al. 2014). Conversely, plant- and insect-associated microbes can also strongly affect the performance of herbivorous insects on a shared host plant either directly, as endosymbionts, entomopathogens (Shikano et al. 2017) or as a source of nutrition for the insect (Hatcher 1995), or indirectly, through modification of the plant's nutritional or defense status (Lazebnik et al. 2014, Biere and Goverse 2016). Finally, plant composition in turn can shape the interactions between insects and insect pathogens by modulating the nutrient intake of the insect, or by providing secondary metabolites that enhance or weaken the insect's ability to resist pathogens (reviewed by Cory and Hoover 2006).

While there is extensive literature on plant-mediated effects of beneficial plant microbes on insect performance (e.g. mycorrhizae and plant growth promoting rhizobacteria; Pineda et al. 2010, Jung et al. 2012), fewer studies have addressed the effects of plant pathogens (i.e. phytopathogens; reviewed in Tack and Dicke 2013, Fernandez-Conradi et al. 2017). For example, the activation of plant defenses by a phytopathogen infection can in turn influence the performance of an insect attacking the plant (reviewed by Stout et al. 2006, Lazebnik et al. 2014). Moreover, phytopathogens may interfere with insect performance also directly by using insects as alternate hosts or primary vectors (Kluth et al. 2002, Belliure et al. 2005, Nadarasah and Stavrinides 2011, Stafford-Banks et al. 2014). A recent meta-analysis shows that, overall, fungal phytopathogens tend to reduce the performance of insect herbivores on plants (Fernandez-Conradi et al. 2017).

One crucial plant-mediated factor shaping herbivore performance is the production of secondary metabolites. Secondary metabolites are organic compounds not directly involved in plant primary metabolism, but crucial for defense and reproduction (Wink 1988, Dobler et al. 2011, Richardson et al. 2015). However, specialist herbivores have often evolved mechanisms to detoxify or sequester them, and use them to in turn defend themselves from predators (reviewed by Després et al. 2007, Opitz and Müller 2009). However, physiological costs of feeding on defended plants, such as reduced survival and lifespan, have also been

reported in specialized herbivores (Camara 1997, Tao et al. 2016).

Studies on insect dietary composition generally assess insect performance in terms of development and survival. However, the ability to mount an immune response is equally relevant in shaping insect performance, as insects themselves face pathogens and parasites. Insect immunity can vary with diet quantity (Siva-Jothy and Thompson 2002) and quality (reviewed by Lampert 2012), both in terms of nutrient content (Lee et al. 2006) and concentration of plant secondary metabolites (Martemyanov et al. 2012, de Roode et al. 2013, Tao et al. 2016). An optimal diet is expected to minimize the costs of activating an immune response by reducing the physiological tradeoffs between investment in immunity and other life-history traits (Sheldon and Verhulst 1996). Indeed, costs related to the activation of the immune system alone have been reported (Schmid-Hempel 2005, Rolff and Reynolds 2009). For example, uncontrolled immune responses can lead to the production of compounds that are toxic for the insect (Cerenius et al. 2008), hence the immune system is expected to be activated prevalently by actual threats.

The Glanville fritillary butterfly *Melitaea cinxia* (Lepidoptera: Nymphalidae), is specialized on host plant species containing high concentrations of the secondary metabolites iridoid glycosides (IGs), such as *Plantago lanceolata* (Duff et al. 1965, Adler et al. 1995). Iridoid glycosides act as feeding stimulants for specialist insects and can be sequestered by larvae of checkerspot butterflies (Bowers 1983, Gardner and Stermitz 1988), including *M. cinxia* (Suomi et al. 2003). In *M. cinxia*, high levels of IGs are associated to faster larval development (Saastamoinen et al. 2007), and appear to act as oviposition cues for mothers (Nieminen et al. 2003). Previous work has reported the upregulation of a component of insect immunity (i.e. encapsulation rate) by one specific IG in late larval instars (Laurentz et al. 2012). Hence, dietary IGs appear to improve at least some aspects of specialists' performance. Conversely, consumption of host plants infected by the obligate biotroph powdery mildew *Podosphaera plantaginis*, is known to reduce larval growth and overwintering survival (Laine 2004). *Podosphaera plantaginis* is specialized on *P. lanceolata*, and variation in its occurrence shapes plant-pathogen coevolutionary dynamics (Laine 2005), also influencing butterfly population dynamics (Laine 2004). However, its role in shaping insect immunity remains unexplored. We investigated how a phytopathogen can modulate the effects of dietary iridoid glycosides on larval performance and immunity of a specialist herbivore and hence ask: 1) whether the commonly found positive effects of dietary IGs on larval development are reduced by a phytopathogen infection, and 2) whether tradeoffs due to a suboptimal diet arise between larval life-history and investment in immunity, here measured as phenoloxidase activity and the expression of seven immune genes. We expect a superior performance and immune regulation on the 'optimal' diet (i.e. healthy plants with high IGs; Laine 2004, Saastamoinen et al. 2007). Tradeoffs reflecting physiological costs (Sheldon and Verhulst

1996, Zuk and Stoehr 2002) of investment in immunity under suboptimal conditions are instead expected especially in larvae reared on infected plants with low IGs.

## Material and methods

### Study species

The Glanville fritillary butterfly in Finland occurs in the Åland Islands in a naturally fragmented habitat consisting of pastures and dry meadows with one of two host plants, *Veronica spicata* and *Plantago lanceolata* (Ojanen et al. 2013). The butterfly has a univoltine life cycle in Finland, with females ovipositing in late June, and the early larval stages are tightly dependent on the oviposition choice made by female. Gregarious larvae enter diapause in September during the fourth or fifth larval instar, and emerge during the following spring (Ojanen et al. 2013).

*Plantago lanceolata* is a perennial rosette forming flowering plant present in Åland, which is occasionally infected by the specialist powdery mildew *Podosphaera plantaginis* (Laine 2004). The pathogen infection reduces plant growth and biomass without killing it (Bushnell and Allen 1962). Approximately 5% of the habitat patches with *P. lanceolata* have the pathogen present each year (Laine 2005), and many of these habitats are also occupied by *Melitaea cinxia*. The local mildew infection in the Åland Islands starts towards the end of the oviposition season of *M. cinxia*, and peaks when the larvae are about to enter diapause (Laine 2004).

### Experimental design

*Plantago lanceolata* plants artificially selected to contain high or low constitutive leaf concentrations of iridoid glycosides (i.e. aucubin and catalpol) were used in the experiment (selected by A. Biere and H. B. Marak at the Netherlands Inst. of Ecology; Marak et al. 2000). The selection on leaf IGs resulted in no correlated variation in leaf content of primary metabolites (N, P, K; Biere et al. 2004). The 60 plants used in the experiment consisted of five families of high (H4, H6, H7, H8, H10) and four families of low iridoid lines (L1, L3, L8, L9). The content of aucubin and catalpol was measured with nuclear magnetic resonance (NMR) technology following methods by Kim et al. (2010), to confirm the differences in IG levels between the lines (Supplementary material Appendix 1). Half of the plants per family were inoculated with a mix of two natural strains of powdery mildew from Åland by brushing mildew spores on the central leaves of the rosette 15 days before the experiment started, to allow the fungal infection to spread (Laine 2005). All the inoculated plants showed visible signs of infection throughout the experiment. All plants were kept in climate chambers at constant temperature and light regime (23:20°C; 18:6h, L/D).

Larvae used in the experiment originated in captivity from parents collected as diapausing larvae from 38 populations across the Åland Islands. The parent generation was reared

in the laboratory on *P. lanceolata* leaves. The plants were grown from commercial seeds. Egg clutches from 30 freely-mated females were used in the experiment and kept at constant temperature regime until hatching (28:15°C; 12:12h, L/D). During the first instar, larval groups were reared on commercial *P. lanceolata* leaves. Larvae of each family were haphazardly divided into eight petri dishes corresponding to eight treatment groups and reared under constant conditions until diapause (23:20°C; 18:6h, L/D), except for two families, which were more numerous and thus divided into nine groups (i.e.  $n = 242$  larval groups in total). The treatment groups resulted from a full factorial design among the following: diet with high or low IG content, and with or without mildew infection, and larval immune challenge with bacteria or a control solution. Plants within each treatment (control versus mildew) were divided between two climate chambers (i.e. four climate chambers in total) and randomized within and between chambers daily. Larval groups were fed daily with fresh leaves, and monitored for development and survival. To eliminate biases to a specific plant family, larvae were fed a mixture of leaves from different families. Leaves were offered until all the larvae in a petri dish reached the 5th and diapausing instar and terminated feeding spontaneously. As *M. cinxia* larvae are gregarious, development could not be followed individually, hence all the variables measured are averages among siblings within each petri dish.

Once all the larvae in a petri dish entered the diapause they were individually weighed. To challenge the immune system, one half of the petri dishes underwent a bacterial immune challenge via puncturing of individual larvae with a microscopic needle (diameter 2–10  $\mu\text{m}$ ) dipped in a solution containing 200  $\text{mg ml}^{-1}$  of the non-pathogenic bacterium *Micrococcus luteus* (lyophilized cells ATCC no. 4698) in PBS (phosphate-buffer-saline solution). This was done 24 h after all the siblings in a petri dish had entered diapause, as introducing an immune challenge earlier could have impaired larval development, or caused the experiment to end before growth differences due to the diet could be detectable. The remaining groups underwent this treatment with PBS alone as wounding control. For logistic reasons, we could not include a naïve control treatment. However, previous studies have shown that wounding has only moderate effects on the tested immune genes (Woestmann et al. 2017). Larvae were snap-frozen in liquid nitrogen 24 h after the immune challenge, and randomly divided into two groups: three larvae/petri dish for RNA extraction (immune gene expression) and the remaining larvae for phenoloxidase activity immune assay, both stored at  $-80^{\circ}\text{C}$  for later analysis.

### Phenoloxidase activity

One  $\mu\text{l}$  of hemolymph was sampled from a pool of three siblings per petri dish, mixed with 23  $\mu\text{l}$  of ice-cold PBS, and stored at  $-80^{\circ}\text{C}$  for later analysis. Two biological replicates, hence six siblings per petri dish, were used for this immune assay. Phenoloxidase (PO) activity was assessed with modified methods by Freitak et al. (2007). Hemolymph samples



were thawed, centrifuged at 13 000 rpm and 4°C, and the assays were performed in 96-well plates. To assess PO activity, 7.5 µl of supernatant were mixed with 70 µl of ice cold MilliQ water, 10 µl of ice cold PBS and 10 µl of ice cold 10 mM L-Dopa solution, and changes in absorbance were read at 30°C every minute at 490 nm with an EnSpire microplate reader. Kinetic activity was measured as the slope of the reaction curve during the linear phase ( $V_{\max}$ : linear phase between 5 and 11 min). The average of the two replicates was used as measure of PO activity per petri dish.

## Immune gene expression

RNA extraction from a pool of three frozen larvae, and cDNA synthesis were performed following methods by Woestmann et al. 2017 (Supplementary material Appendix 2).

### Real-Time qPCR

We used primers for seven immune genes associated to the Toll and Imd pathways, responsible for pathogen-defense in insect immunity (Rolff and Reynolds 2009; Toll: lysozyme, prophenoloxidase (proPO),  $\beta$ -1,3-glucan recognition protein ( $\beta$ GRP), pelle and serpin 3a; Imd: attacin and peptidoglycan recognition protein LC (PGRP-LC), and two housekeeping genes (histone H2A.Z and mitochondrial ribosomal protein L37), designed by Woestmann et al. (2017). The genes have functions in butterfly immunity analogous to the ones in *Drosophila* (Cao et al. 2015), and were chosen in order to cover a wide range of immune responses involved in the processes of pathogen recognition ( $\beta$ GRP, PGRP-LC), cellular immunity (proPO and its inhibitor serpin), humoral immunity and production of antimicrobial peptides (pelle, attacin, lysozyme). These genes have been previously shown to respond to bacterial exposure in *M. cinxia* (Woestmann et al. 2017), while the Toll pathway is in general expected to react to fungi (Rolff and Reynolds 2009). The qPCR was performed with a C1000 Thermal Cycler on 384-well plates with three technical replicates of 1:5 diluted cDNA, one negative control, and one plate control sample. Each well included 1 µl of cDNA, 0.5 µl of each primer (10 µM) (Oligomer Oy), 5 µl of iQ SYBR green Supermix for qPCR, 3 µl of MilliQ water. Control-RT samples were also created to rule out any contamination with genomic DNA.

## Statistical analysis

Median values per plant genotype of NMR metabolite quantifications (percentage of dry weight) were square root transformed to normalize their distributions. We used as response variable both the sum of aucubin and catalpol concentration and the individual amounts of aucubin and catalpol. For each response variable we built a linear model including whether the plant genotypes had been selected for high or low IG content.

To assess the effect of diet treatment on the development of the larval groups, larval growth rate was calculated dividing the average body mass of the diapausing instar (mg) by the average development time until diapause (d) of each petri dish. Growth rate was analyzed with a linear mixed model approach including iridoid (high versus low) and mildew (presence versus absence) treatments and their interaction as fixed factors, and larval family as random effect. Survival of sibling groups was analyzed with a generalized mixed model with binomial error distribution and the same fixed and random factors as above. To assess if larval immune response varied with diet, we additionally included bacterial treatment in the mixed models for PO activity and immune gene expression and all two and three-way interactions with IG and mildew treatments. The expression of immune genes was calibrated to individuals fed on low IG plants in the absence of mildew and experiencing the wounding control, and the analysis was performed separately for each gene. Finally, we calculated the proportion of variance explained by the larval family random term for all the response variables listed above as intraclass correlation coefficient (ICC) of the model explaining each response variable (Table 1).

All data were analyzed with R for Windows, ver. 3.3.2 (<[www.r-project.org](http://www.r-project.org)>)(packages ‘MASS’ and ‘lme4’; Venables and Ripley 2002, Bates et al. 2015).

## Data deposition

Data available from the Dryad Digital Repository: <<http://dx.doi.org/10.5061/dryad.td0j2r8>> (Rosa et al. 2018).

## Results

### NMR IGs quantification

Both the cumulative IG content ( $F_{1,7} = 35.98$ ,  $p = 0.0005$ ) and the individual quantifications of aucubin ( $F_{1,7} = 23.09$ ,  $p = 0.002$ ) and catalpol ( $F_{1,7} = 25.99$ ,  $p = 0.001$ ; Supplementary material Appendix 2 Table A1) were markedly higher in the lines selected to contain high IGs.

Table 1. Proportion of variance explained by the larval family random term, calculated as intraclass correlation coefficient (ICC) of the model explaining each response variable.

Response variable	Family ICC (%)
Growth rate	66.65
Larval survival	19.51
PO activity	9.06
Lysozyme	13.17
proPO	28.44
PGRP-LC	33.48
$\beta$ GRP	22.22
Attacin	26.30
Serpin	7.14
Pelle	25.26

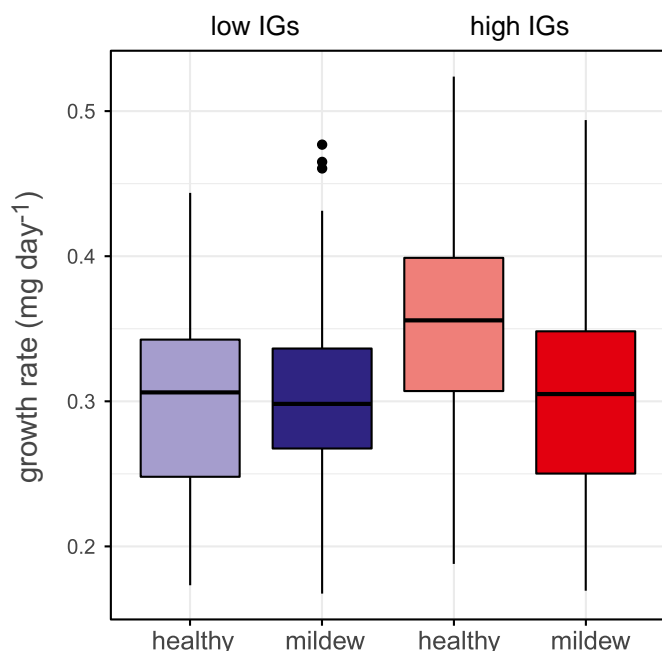


Figure 1. Variation in average larval growth rate in relation to iridoid glycosides content and mildew presence in the diet. Blue and red color represent low and high IGs, and dark shading represents mildew presence, respectively.

### Larval performance

Larval growth rate was reduced by low IGs ( $F_{1,209} = 24.97$ ,  $p < 0.0001$ ) and mildew ( $F_{1,209} = 24.73$ ,  $p < 0.0001$ ; Supplementary material Appendix 2 Table A1). Effects of mildew infection on larval growth rate depended on IG content; whereas mildew strongly reduced larval growth rate in high IG plants, it did not in low IG plants (IG  $\times$  mildew,  $F_{1,209} = 29.95$ ,  $p < 0.0001$ ; Fig. 1). Approximately 86% of the larvae survived until diapause, with no effect of mildew infection ( $\chi^2 = 1.16$ ,  $p > 0.2$ ). However, larval groups with high mortality were more frequent in the high IG treatment

( $\chi^2 = 8.99$ ,  $p < 0.003$ ; Supplementary material Appendix 2 Fig. A1). Notably, larval family explained more than 66% of the variance in the growth rate, and 19.5% of the average survival (Table 1).

### Immune response

The PO activity was lower in larvae challenged with bacteria ( $F_{1,204} = 15.1$ ,  $p = 0.0001$ ; Fig. 2a). Furthermore, PO activity was marginally lower in the high IG treatment group and lower in larvae fed on mildew-infected plants (IGs,  $F_{1,204} = 3.6$ ,  $p = 0.06$ ; mildew,  $F_{1,204} = 5.4$ ,  $p = 0.02$ ; Fig. 2b–c). No significant second or third order interactions were detected ( $p > 0.2$  for all).

The largest effect on immune gene expression was due to bacterial challenge, affecting five out of seven immune genes. Bacterial challenge upregulated attacin ( $F_{1,205} = 365.4$ ,  $p < 0.0001$ ),  $\beta$ GRP ( $F_{1,204} = 98.8$ ,  $p < 0.0001$ ), lysozyme ( $F_{1,206} = 10.9$ ,  $p = 0.005$ ), and pelle ( $F_{1,204} = 48.5$ ,  $p < 0.0001$ ), and downregulated proPO gene expression ( $F_{1,205} = 7.3$ ,  $p = 0.008$ ; Fig. 3a, Supplementary material Appendix 2 Table A2). No main effect of IG diet was detected in any of the genes ( $p > 0.1$  for all, Fig. 3b). An interaction suggested that the upregulation of pelle in the bacterial treatment was more pronounced in larvae fed on high IGs ( $F_{1,204} = 5.0$ ,  $p = 0.03$ ; Supplementary material Appendix 2 Table A1). A similar trend was found for  $\beta$ GRP ( $F_{1,203} = 3.5$ ,  $p = 0.07$ ). Another interaction suggested that attacin was upregulated in larvae fed on high IG plants infected with mildew (IGs  $\times$  mildew,  $F_{1,205} = 4.4$ ,  $p = 0.04$ ). Mildew infection alone upregulated gene expression of two genes, lysozyme and proPO ( $F_{1,206} = 4.4$ ,  $p = 0.04$ , and  $F_{1,205} = 7.2$ ,  $p = 0.008$ , respectively; Fig. 3c). Moreover, a more pronounced attacin upregulation was evident in bacteria-challenged larvae fed on mildew-infected plants (mildew  $\times$  bacteria,  $F_{1,205} = 5.0$ ,  $p = 0.03$ ; Supplementary material Appendix 2 Table A1). We found no effect of any of the variables tested on PGRP-LC and serpin gene expression ( $p > 0.1$  for all). Finally, the proportion of variance explained by larval family was 9% for

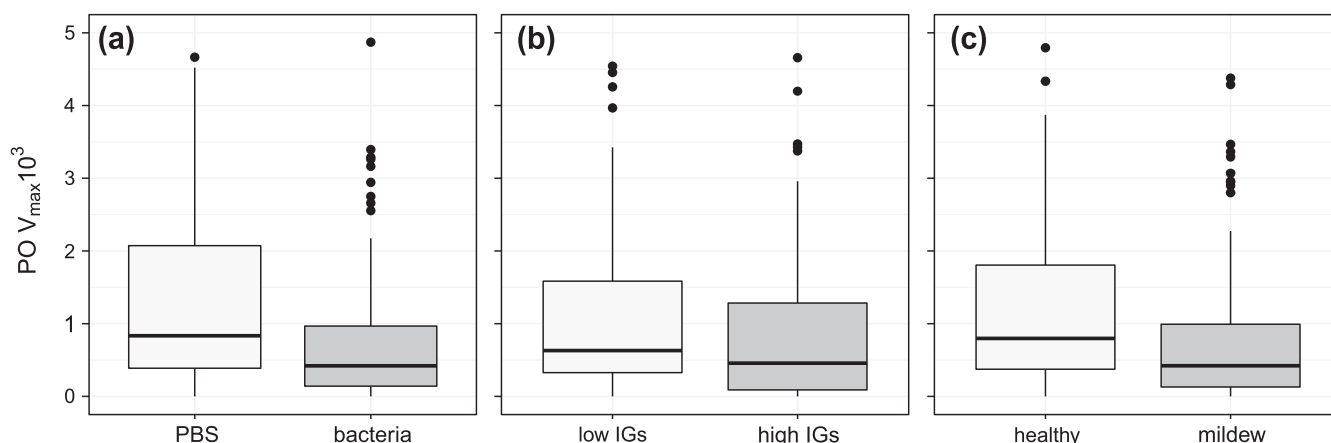


Figure 2. Changes in larval phenoloxidase activity during the linear phase ( $PO V_{max} \times 10^3$ ) for bacterial challenge (a), iridoid glycosides (b) and mildew in the diet (c). Dark grey fill represents bacterial challenge (a), high IGs (b), and mildew presence (c).

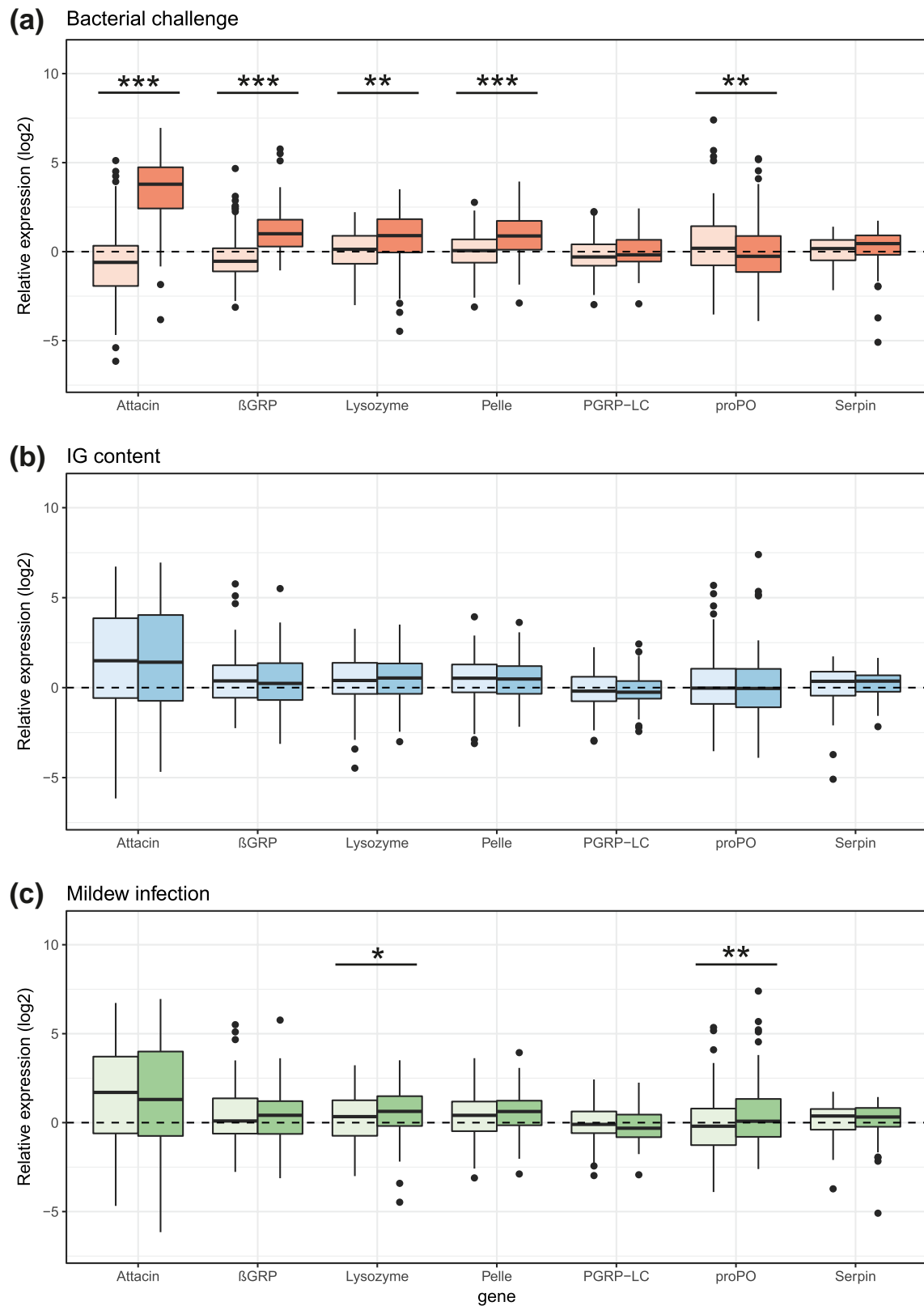


Figure 3. Relative immune gene expression ( $\log_2$ ) upon wounding or bacterial challenge (a), high or low IG content (b), and mildew absence or presence in the diet (c). Dark fill represents bacterial challenge (a), high IG content (b) and mildew presence (c).

the PO activity and ranged from 7% to over 33% for the immune genes, with proPO, PGRP-LC and attacin having the highest ICC values (Table 1).

## Discussion

Tripartite interactions among plants, phytopathogens and insect herbivores critically modulate the fitness of all three players. Here, we focused on the performance and immune response of a specialist insect herbivore feeding on infected host plants. While confirming previous findings showing a positive effect of high IGs and a negative effect of mildew infection on larval growth (Laine 2004, Saastamoinen et al. 2007), we further show that infection by a phytopathogen can minimize the benefits of dietary IGs. The phytopathogen also had contrasting effects on insect immunity by reducing PO activity while upregulating the expression of two immune genes. The high IG diet had a role in upregulating two immune genes, but only in interaction with a bacterial challenge and mildew presence, respectively. Also, PO activity was marginally lower and survival was reduced in the high IG treatment, indicating a possible cost of sequestering or detoxifying secondary metabolites.

Our results indicate that both larval growth and immune response are highly sensitive to host plant condition. While the effects of IG dietary content on immunity seem mostly dependent on specific conditions, the main effects caused by mildew presence on larval immunity deserve further attention.

### Effects of diet on larval performance

We measured the content of iridoid glycosides and confirmed a markedly higher concentration in the families selected to contain high IGs. Despite growth rate varying considerably among larval families (Table 1), both mildew and IG treatments substantially impacted larval growth (consistent with Laine 2004, Saastamoinen et al. 2007). As expected, larvae feeding on the 'optimal' diet (healthy plants with high IGs) had the highest growth rate, as found with other specialist lepidopterans (Bowers 1983, 1984). Conversely, both mildew and low IGs reduced larval growth, and the three 'sub-optimal' diets all led to equally poor larval performances (Fig. 1). Indeed, a mildew infection considerably altered the impact of high IGs on larval performance by removing their positive effect on growth rate. Whether this is caused by the mildew infection reducing the levels of IGs produced by the host plants or by mildew infection modulating the effect of a given level of IGs on larval growth should be formally investigated. However, the content of IGs in the selected families that we used did not show a detectable change following mildew infection (Supplementary material Appendix 3 Table A3–A4). Hence, we suggest that mildew infection mitigated the effects of IGs on larval growth, rather than that it changed the amount of IGs produced by the plant. It is also possible that the effects are mediated via changes in other essential

nutrients. In contrast to larval growth, larval survival was not significantly related to the presence of mildew. Instead, larval groups with higher mortality were found more frequently in the high IG treatment (Supplementary material Appendix 2 Fig. A1), suggesting a possible cost of metabolite sequestration or detoxification, similar to what found for example with the buckeye with IGs (Camara 1997) or monarch butterflies with cardenolides (Tao et al. 2016).

### Effects of diet on immunity

A bacterial immune challenge considerably altered immune gene expression. Four out of seven genes were upregulated by bacterial challenge, three of which associated with the Toll pathway, normally triggered by gram-positive bacteria including *Micrococcus luteus* (Madigan et al. 2006, Rolff and Reynolds 2009). Furthermore, the activation of genes associated to the JAK-STAT pathway, involved in stress and injury responses (Rolff and Reynolds 2009), cannot be ruled out. Attacin is instead controlled by the Imd pathway, normally triggered by gram-negative bacteria (Rolff and Reynolds 2009), but it also showed increased expression levels upon a gram-positive bacterial challenge. Nonetheless, Toll and Imd are interacting (Rolff and Reynolds 2009), and cross-talk between the two pathways may potentially explain the upregulation of attacin. Contrarily to the other Toll-associated genes tested, proPO was downregulated by bacterial immune challenge, and so was the PO activity itself. PO activity can be upregulated as part of the immune defense of insects, but it produces, as an outcome, reactive oxygen species (ROS) that are toxic to insects and, thus, this defensive pathway is normally tightly regulated (Cerenius and Söderhäll 2004).

A high content of dietary IGs had no clear main effect on immunity, except for a marginal reduction of PO activity. This result is similar to findings with other lepidopterans specialized on IGs (i.e. melanization response; Smilanich et al. 2009, Richards et al. 2012, Lampert and Bowers 2014), and has been interpreted as a tradeoff between investment in IG sequestration/detoxification and immune defense (i.e. 'vulnerable host hypothesis'; Smilanich et al. 2009). We did, however, find some evidence of immunity being upregulated by high dietary IGs in the presence of a bacterial challenge (pelle, and BGRP marginally), and in the presence of mildew (attacin). A positive role of dietary IGs in the immunity of *Melitaea cinxia* has been previously reported in terms of higher encapsulation rate in the presence of dietary catalpol alone (Laurentz et al. 2012). Our data further support the idea that dietary IGs play a role in modulating immune defenses. However, we suggest that the outcome is context-dependent, and should not be generalized, but rather be related to the specific type of microbe and component of immunity tested.

Interestingly, the presence of the fungal pathogen on the host plant also upregulated the expression of two genes of the Toll pathway: lysozyme and proPO. In addition, attacin was upregulated by mildew on the host plant only in the presence of a bacterial challenge. The upregulation of immune genes in larvae fed on the diets including mildew may be explained



either by the mildew being detected by the insect immune system as a potential threat, or by the mildew causing some alterations in the host plant and indirectly activating the insect immune system. Mildew infection has been shown to alter plant primary metabolism (i.e. nitrogen, carbohydrates and water content; Allen 1942, Bushnell and Allen 1962, Walters and Ayres 1983) and induce production of defense hormones (e.g. jasmonic acid, salicylic acid, ethylene), secondary metabolites (e.g. defensins or proteinase inhibitors) or a combination of these in several plant species (reviewed by Stout et al. 2006), which all may indirectly affect insect immunity. On the other hand, the Toll pathway is associated specifically with fungal recognition (Rolff and Reynolds 2009), hence mildew infection of the host plant could also directly affect *M. cinxia*'s immunity. Nonetheless, cases where insect immunity has been affected by ingestion of non-entomopathogenic generalist (Freitak et al. 2007, 2009) and plant-specific microbes (Shikano et al. 2015, Olson et al. 2017) have been reported in literature. In addition, cases of insects becoming infected with phytopathogens or acting as transmission vectors have also been reported (Kluth et al. 2002, Medeiros et al. 2004, Nadarasah and Stavrinides 2011). The upregulation of immune genes in larvae fed on the mildewed diet could also reflect a general stress response, as found with forced flight upregulating some of the immune genes tested here (Woestmann et al. 2017). Similarly, the larvae may be more susceptible to microbes due to the negative effects the mildewed diet had on their growth (Rostás and Hilker 2003). In any case, this probably unnecessary investment in immunity is likely to be costly (Zuk and Stoehr 2002) and potentially maladaptive, and, as such, may partially explain why larvae fed on mildew-infected leaves showed reduced growth. Costliness of immune activation is further supported by the PO activity itself being marginally lower in larvae fed on the mildew-infected diet. One possible interpretation of this may be a mismatch between immune activation induced by the fungus presence on the plant inducing the gene expression, and a physiological cost on the larvae failing to accumulate the amino acids required as substrate for the PO enzyme when feeding on a diet with potentially low nutritional value, like the one with the mildew (Cerenius and Söderhäll 2004). The attacin upregulation only in presence of both mildew infection and bacterial challenge could be interpreted as a priming effect (i.e. a preventive immune activation), potentially caused by the mildew ingested before the bacterial challenge (Little and Kraaijeveld 2004). It would be interesting to test whether presence of mildew or some plant symbionts could act as a form of self-medication on insect herbivores against bacterial infections. Finally, the proportion of variance explained by family for PO activity and immune gene expression was moderate, suggesting a marginal role of genetic background in shaping immune responses.

### Ecological and evolutionary implications

The observed effects of powdery mildew infection on larval growth rate and immunity raise questions on the potential

ecological and evolutionary implications. First, are mildew effects on larval performance likely to affect the distribution of disease and/or herbivory in the (meta)population? As mildew infections are generally not apparent by the time female butterflies make their oviposition host decision, the potential effects of mildew infection on the distribution of *M. cinxia* among host plants are likely to be mediated by larval movement patterns rather than by female oviposition decisions. Based on the observed effects of mildew, such as counteraction of positive IG effects on larval growth and probably costly induction and priming of the larval immune response, larvae are expected to move away from mildewed to mildew-free plants, whenever possible. Indeed, previous work by Laine (2004) has shown that, given the option, larvae are more likely to move away from mildew-infected than from healthy plants. However, once they have left the initial mildew-infected plant, they accept infected and uninfected plants with equal probability upon first encounter (Laine 2004). This is likely caused by the fact that pre-diapause larvae generally have low mobility, limiting host plant choice, consequently preventing them from specifically shifting onto mildew-free plants. The reduced mobility of the larvae is also likely to limit their role as vectors of the disease, spores of which are typically wind-dispersed (Laine and Hanski 2006). It would be interesting to test, however, whether adults that as larvae fed on mildew-infected hosts display increased dispersal from the original patch with the pathogen to a new, pathogen-free area (Matthysen 2012), as an adaptive plastic response.

Another interesting question regarding implications is whether the effects of mildew on larval growth and immunity potentially alter selection pressure on larval secondary compound sequestration strategies. Mildew infection negatively interacts with the beneficial effects of high IGs on larval growth. Our data do not indicate that this would be due to a substantial reduction in the amount of IGs accumulated by the host plant following a mildew infection. However, it is possible that mildew ingestion reduces the ability of larvae to detoxify or sequester these metabolites. Mildew presence could also affect levels of phytohormones involved in the signaling and activation of plant defense (Stout et al. 2006, Lazebnik et al. 2014), which may influence IG sequestration. Biotrophic pathogens generally activate the salicylic acid defense signaling pathway. In several species, negative cross-talk has been shown between the salicylic acid defense signaling pathway and the jasmonic acid pathway that is involved in activating defenses against chewing herbivores (Ponzio et al. 2013). Consequently, if levels of IG sequestration are proportional to tissue levels of IGs, and the production of IGs is activated downstream of jasmonic acid signaling, biotrophic pathogens such as powdery mildew may potentially lead to reduced levels of IG sequestration by the larvae. Reduced IG-sequestration ability might select for strategies to feed on plants with even higher IG content in mildewed populations with high incidence of parasitoids or predators to mitigate the negative effects of mildew on the efficacy of IG sequestration. Future studies should assess in more detail the role

of mildew infection in shaping butterfly occupancy and the multi-trophic dynamics within the Åland meadow network.

## Conclusions

We found that high IG content is a clear predictor of a better insect growth in the absence of a phytopathogen, while a mildew infection can substantially minimize the positive effects of dietary IGs on larval growth. However, the ingestion of dietary IGs may come with a cost in terms of survival, and possibly PO activity, potentially due to an excessive energetic demand of sequestering or detoxifying the metabolites. Yet, dietary IGs also upregulated immune gene expression in a context-dependent way, emphasizing the complexity of insect immunity and the importance of assessing immune defense from different angles. Notably, we showed that the presence of a plant pathogen can induce insects to allocate costly resources to upregulate their immunity, which could lead to physiological tradeoffs and may partially explain the reduced fitness of larvae fed on a mildew-infected host. It remains unclear whether these effects result from a direct interaction between mildew and the larvae or via alternative indirect effects. Nonetheless, interactions between insect herbivores and phytopathogens have been reported to take place more frequently than assumed (Shikano 2017), opening an exciting perspective or future research on multi-trophic interactions.

**Acknowledgements** – We would like to thank Anna-Liisa Laine and Krista Raveala for providing the mildew strains, Juha-Matti Pitkänen for setting up the experiment and performing the NMR analysis, Elisa Metsovuori for helping during the experiment, Toshka Nyman for RNA extractions, Laura Häkkinen for performing the PO assay, and Guillaume Minard for sharing his unpublished data. We also thank Sara Magalhães for comments on a previous version of the manuscript.

**Funding** – This work was supported by Kone Foundation, Academy of Finland (grants 284601 and 304041) and the European Research Council (Independent StG grant; META-STRESS 637412). The authors declare no conflict of interest.

**Author contributions** – ER and MS conceived the ideas and experimental design. LW designed qPCR methodology and analysis. AB provided the plant material for the experiment. ER collected and analyzed the data. ER, AB, LW and MS all contributed to the writing of the manuscript.

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Supplementary material (available online as Appendix oik-05437 at <[www.oikosjournal.org/appendix/oik-05437](http://www.oikosjournal.org/appendix/oik-05437)>). Appendix 1–3.